



Evolution of Developmental Control Mechanisms

Predetermination of sexual fate in a turtle with temperature-dependent sex determination

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ABSTRACT

Egg incubation temperature determines offspring sex in many reptilian species, including red-eared slider turtles, where embryos incubated at low temperatures during the initial stages of gonad formation develop as males, while those kept at higher temperatures develop as females. Incubation at the threshold, or pivotal, temperature (PvT) yields an even ratio of males and females. This strong susceptibility to temperature indicates that each embryo of this species is competent to develop as a male or a female. However, the mechanism that determines sexual fate at the PvT has not been identified. One possibility is that sexual fate is stochastic at the PvT, but coordinated by systemic signals within a single embryo. If this is the case, gonads explanted separately to culture should not coordinate their fate. Here we show that gonad pairs from embryos incubated at the PvT share a strong predisposition for one sex or the other when cultured in isolation, indicating that they were affected by shared genetic signals, maternally-deposited yolk hormones or other transient influences received prior to the stage of dissection. *In ovo* studies involving shifts from the male- or female-producing temperature to the PvT further indicate that embryos adopt a sexual differentiation trajectory many days prior to the onset of morphological differentiation into testes or ovaries and usually maintain this fate in the absence of an extreme temperature signal favoring the development of the other sex. Our findings therefore suggest that the outcome of sex determination in these reptiles is heavily influenced (i) by an inherent predisposition at the PvT and (ii) by the sexual differentiation trajectory established early in gonad development under male- or female-producing temperatures.

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Introduction

Unlike mammals and birds, which exhibit stable systems of genetic sex determination (GSD) and highly differentiated sex chromosomes, reptiles have evolved an extraordinary array of sex-determining mechanisms (Janzen and Phillips, 2006), with many species relying on environmental rather than genetic signals to determine offspring sex. Many different types of male (XX/XY) and female (ZZ/ZW) heterogamety have been reported among GSD reptiles, with different pairs of autosomes serving as the sex chromosomes and with varying levels of heterogametic differentiation (Pieau et al., 1999; Graves and Shetty, 2001; Kawai et al., 2007; Badenhorst et al., 2013). However, in all crocodilians so far tested and many turtles and lizards, embryonic sex is regulated by the incubation temperature of the egg during the middle third of development when the gonads are forming

(temperature-dependent sex determination (TSD)) (reviewed by Pieau et al. (1999)).

Phylogenetic analyses indicate that the ancestral sex-determining mechanism in the vertebrate lineage was GSD, so a transition from GSD to TSD must have occurred at least once during reptilian evolution (Janzen and Krenz, 2004). Further evidence indicates that multiple independent transitions from TSD back to GSD subsequently took place as reptiles diverged (Janzen and Krenz, 2004; Janzen and Phillips, 2006; Pokorna and Kratochvil, 2009). Retention of some degree of thermosensitivity in GSD species may underlie these evolutionary transitions (Quinn et al., 2011). Indeed, in certain reptiles, egg incubation temperature can override a GSD mechanism, even when heteromorphic sex chromosomes are present. Such a system has been described in the dragon lizard *Pogona vitticeps*, which utilizes a ZZ/ZW GSD system (Ezaz et al., 2005) that can be overridden at high temperatures to produce ZZ females (Quinn et al., 2007). Similarly, the skink *Bassiana duperreyi* exhibits XX/XY heterogamety, but exposure to low temperatures causes XX females to develop as males (Shine et al., 2002; Radder et al., 2008; Quinn et al., 2009). Furthermore, in the GSD turtle *Apalone mutica*, the expression of several genes involved in gonad development is affected by egg

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incubation temperature, again supporting the idea that GSD systems may retain some sex-related thermosensitivity (Valenzuela, 2008). Collectively, these phenomena suggest that the border between TSD and GSD is blurred, and that both systems can, at least in part, stably exist simultaneously (Sarre et al., 2004; Barske and Capel, 2008).

The TSD phenomenon, first reported in a reptile 47 years ago (Charnier, 1966), typically manifests as one of three main patterns: MF or FM, in which one sex is produced at low temperatures and the other at high temperatures, or FMF, in which females develop at both high and low temperatures and males at intermediate temperatures (Ewert et al., 1994). Both sexes are produced at temperatures between the MPT and FPT(s), with the threshold, or pivotal, temperature (PvT) defined as the temperature(s) that produces an average sex ratio of 1:1 (Mrosovsky and Pieau, 1991; Ewert et al., 1994). Functional hermaphroditism has not been reported in reptiles, though in some species, intersex gonads are occasionally observed in hatchlings incubated at the PvT; these typically resolve into testes over time (Pieau et al., 1998). Because of this strong susceptibility to temperature, each embryo of a TSD species is theoretically competent to develop as male or female. However, the mechanism that determines whether an individual will develop as a male or a female at the PvT has not been elucidated.

Sex determination at the PvT could be stochastic. However, the fact that sexual outcomes at the PvT are concordant *in ovo*, i.e., individuals develop either two testes or two ovaries, indicates that development of the two bipotential gonads within a single embryo is coordinated. This could result from shared genetic information or from systemic signals originating from the yolk or the embryo itself. Indeed, it has been proposed that cryptic genetic mechanisms determine sex at the PvT, but are masked at more extreme temperatures (Zaborski et al., 1988; Pieau et al., 1998). Such mechanisms could potentially rely on the cumulative effects of multiple loci across the genome or within a pair of microscopically indistinguishable (homomorphic) sex chromosomes.

The limited evidence for cryptic heterogamety in TSD species largely derives from HY antigen studies performed in the 1980s. HY antigen is a minor histocompatibility marker linked to sex. In mammals, only males are positive for HY antigen, consistent with the fact that several components of the antigen map to the Y chromosome (reviewed by Wolf, 1998). Conversely, among non-mammalian vertebrates with differentiated sex chromosomes, the heterogametic sex tested positive for HY antigen in nearly all species analyzed (Nakamura et al., 1987), i.e., males in species with XX/XY heterogamety and females in species with ZZ/ZW heterogamety. While the nature of HY's involvement in sex determination or differentiation remains unresolved and controversial (Wolf, 1998), HY antigen status has provided a useful way of determining the heterogametic sex in GSD species with homomorphic sex chromosomes (Engel et al., 1981).

Differences in HY status between males and females are less straightforward in TSD species. In *Trachemys scripta*, a small sample of females tested positive for HY antigen in several tissues, suggestive of cryptic ZZ/ZW heterogamety (Engel et al., 1981; Nakamura et al., 1987). However, egg incubation temperature during the TSP was not known for the individuals assayed. More definitive experiments were performed in *Emys orbicularis*. Here, Zaborski and colleagues found that HY status in the gonad reflects phenotypic sex, whereas serological status may reflect genotypic sex (Zaborski et al., 1982, 1988). Under this hypothesis, HY-negative phenotypic females derived from FPT eggs represent thermally sex-reversed genotypic males, whereas HY-positive phenotypic males from MPT eggs represent sex-reversed genotypic females. The authors postulated the existence of a cryptic ZZ/ZW sex chromosome system in *E. orbicularis* (Zaborski et al., 1982),

based on the assumption that HY positivity in blood is associated with the heterogametic sex in TSD turtles as in other vertebrates. However, no follow-up studies have been performed to substantiate these conclusions, and the genetic basis of these patterns remains unclear.

Such cryptic heterogamety or other potential polygenic mechanisms could, in theory, be influenced by temperature during or after an evolutionary transition to or from TSD. Indeed, the coexistence of functional TSD and GSD in a single organism, as observed in the dragon lizard (Quinn et al., 2007), provides an excellent evolutionary framework for transitions between the two systems. However, in *T. scripta*, the existence and/or nature of any genetic factors has remained elusive.

A possible alternative model is that intermediate temperatures result in moderate levels of aromatase expression/activity in the gonads and correspondingly intermediate serum estradiol levels (Pieau et al., 1998). As estrogen is a potent agonist of female development, its uniform global circulation could induce bilateral ovarian differentiation in embryos that meet a hormonal threshold, with male development occurring in those that fall below. Efforts to test the effects of exogenous estrogen on aromatase expression showed no effect at the MPT prior to stage 19, suggesting that the early coordination between left/right gonad development is not regulated by aromatase expression levels (Matsumoto et al., 2013). However, in theory, any circulating substance could serve the function of coordinating left/right gonad development.

In the present study, we explored the question of what underlies the development of males or females at the PvT in the red-eared slider turtle *T. scripta elegans*. We designed a simple experiment in which the two gonads of a given embryo were removed from the body early in the thermosensitive period (TSP) and cultured separately at the PvT. If genetic or systemic factors determine sexual fate prior to this time, then the two gonads should share the same information and therefore develop concordantly when cultured in isolation. If, conversely, the two gonads of a pair do not always adopt the same sexual fate, we could instead conclude that sex is not determined under these conditions by a shared predisposition but rather may require a systemic signal to coordinate the fates of the two gonads.

Materials and methods

Turtle eggs

Freshly laid red-eared slider turtle eggs were acquired from Robert Clark or the Kliebert Turtle & Alligator Farm (Hammond, LA) with the approval of the Louisiana Department of Agriculture and Forestry. In each shipment, we received approximately 500 eggs derived from mixed clutches laid within the same 24-h period. Clutches vary widely in size, but mature *T. scripta* females from different populations have been reported to lay an average of 6.6 to 9.4 eggs per clutch, with some reports of captive turtles laying 15–20, at 12–36 day intervals (Marlen and Fischer, 1999; Aresco, 2004). Assuming a conservative number of 20 eggs per clutch, a shipment of 500 eggs should include eggs from approximately 25 clutches from 25 different females. Based on our sampling simulation studies, in a group of 50 randomly selected eggs, we expect that eight or more would originate from the same clutch less than 1% of the time. We expect this to minimize potential distortions due to clutch variation.

Eggs were incubated in moist vermiculite in a humidified incubator at 26 °C (male-producing temperature, MPT), 31 °C (female-producing temperature, FPT) or 29.2 °C (PvT) with ambient CO₂. Based on experiments using *T. scripta* eggs obtained from

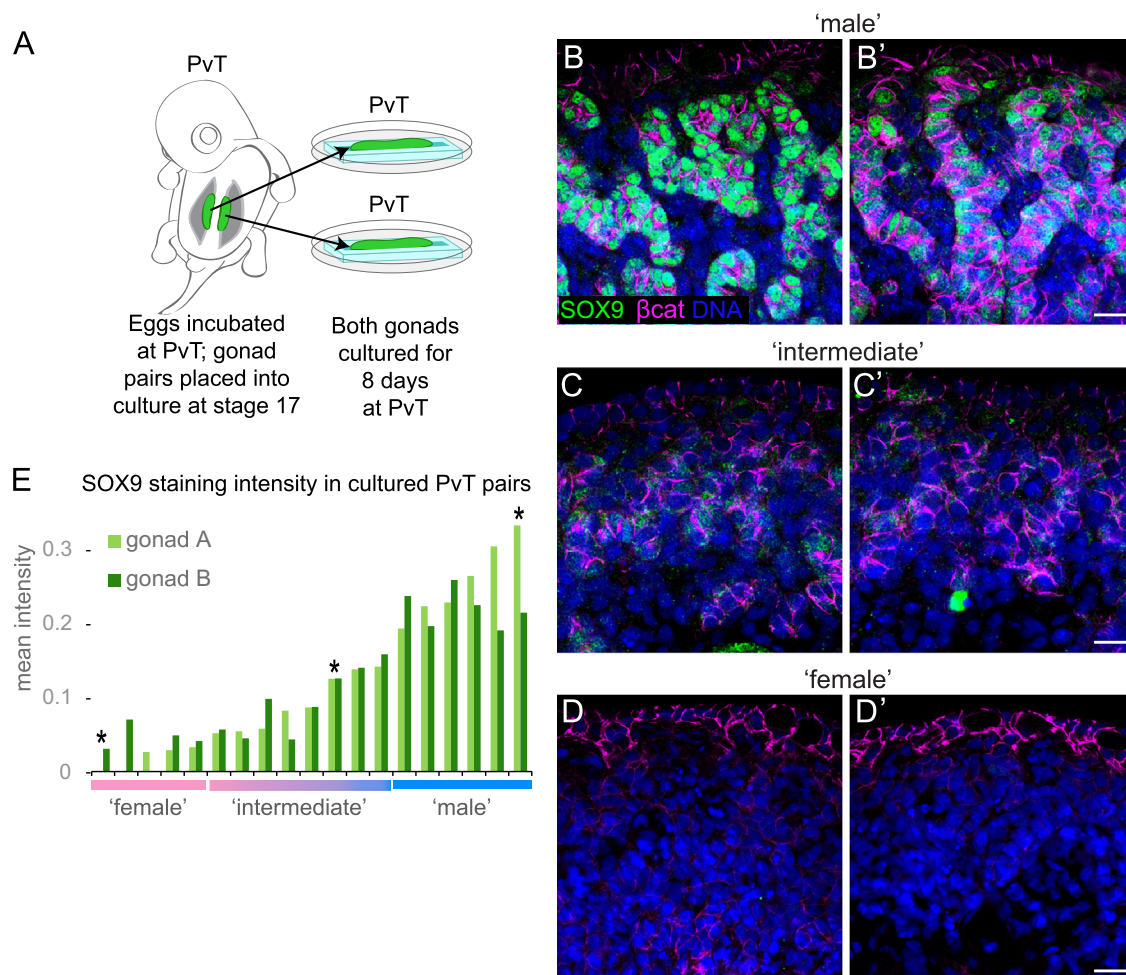


Fig. 1. Sexual fate is synchronized between PvT gonad pairs cultured in isolation. (A) Design of the PvT culture experiment. ((B)–(D)) Pairs of stage 17 gonads cultured individually at the PvT for eight days and then immunostained for SOX9 (green) and β -catenin (magenta). Nuclei were stained with Hoechst (blue). The two gonads of each pair showed very similar staining patterns and morphology. ((B), (B')) Example of a 'male' pair, with high SOX9 expression and clear medullary cord structures enriched for β -catenin. ((C), (C')) Example of an 'intermediate' pair, with low/cytoplasmic SOX9 staining and persistent β -catenin staining in the medulla. ((D), (D')) Example of a 'female' pair, with no apparent SOX9 expression and cortical enrichment of β -catenin. (E) Quantification of SOX9 staining intensity in the 19 surviving gonad pairs cultured at the PvT, ranked according to the value of the first gonad imaged of each pair (i.e., gonad A). The examples shown in ((B)–(D)) are marked with asterisks. Scale bars represent 20 μ m.

Hammond, LA, incubation at 29.2 °C temperature yields an average of 50% male and 50% female embryos across clutches (Wibbels and Crews, 1995), whereas incubation at 26 °C or 31 °C yields 100% males or 100% females, respectively (Bull and Vogt, 1981; Wibbels et al., 1998). Embryos were staged by criteria established by Greenbaum (2002). At stages 17 or 26, they were removed from their eggshells, quickly euthanized and placed into phosphate-buffered saline (PBS) for dissection.

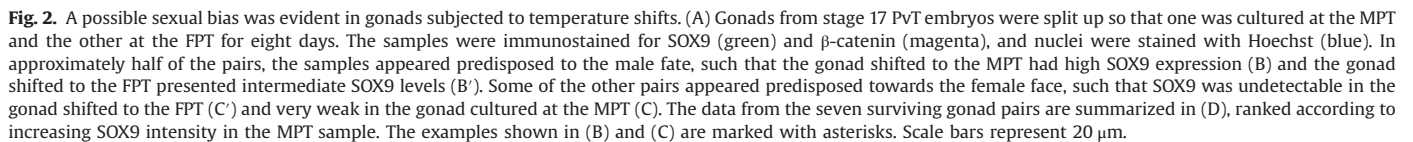
Gonad culture

Eggs were selected randomly for incubation at the PvT upon arrival. Gonad pairs from 30 stage 17 PvT embryos were labeled with a unique identifying number and cultured in separate dishes (Figs. 1 and 2A) using a previously described technique (Mork and Capel, 2013) (adapted from Martineau et al., 1997; Shoemaker-Daly et al., 2010). Briefly, gonads were dissected away from the neighboring mesonephros and laid in thin wells shaped in strips of agar gel (1.5% agar in Leibovitz's L-15 medium (Gibco)) placed in 35-mm tissue culture dishes. Each strip was immersed in 0.3 ml of culture medium comprised of 10% charcoal-stripped FBS in Leibovitz with 50 μ g/ml ampicillin and 1.25 μ g/ml Fungizone (Gibco). One or both of the explanted gonads of 11 embryos developed

fungal infections during the culture period. These pairs were discarded and not included in the analysis. The remaining 19 pairs were cultured at the PvT in ambient CO₂ for eight days (Fig. 1A). In a second small experiment with an initial sample size of eight embryos, one gonad from each pair was shifted to the FPT (31 °C), the other was shifted to the MPT (26 °C), and both were cultured for eight days (Fig. 2A). Seven of these pairs survived the culture period. The gonads were moistened with 5 μ l of medium every day, and the culture medium was changed every other day. On the eighth day, the gonads were removed from the wells, washed in PBS and fixed in 4% paraformaldehyde overnight at 4 °C.

In ovo temperature shift experiment

Eggs were incubated at either the FPT or MPT in groups of 50 until they reached stage 17, as determined by assaying age-matched eggs grown at the same temperature. These eggs were then shifted to an incubator maintained at the PvT, where they remained until stage 26. At this point, each surviving embryo was sexed based on gonadal morphology. A two-tailed binomial test was used to determine significance, with the number of eggs preincubated at the MPT or FPT used as the trial number and with an expected probability of 0.5 for male or female development at



Gonad pairs cultured in isolation at the PvT consistently adopted the same sexual fate (Fig. 1). Strong SOX9 expression was detected in approximately one third of the pairs, indicative of testis fate (Fig. 1B and E), another third exhibited little to no SOX9

expression, indicative of ovary fate (Fig. 1D and E), and the remainder showed intermediate levels (Fig. 1C and E). Of this last group, most gonads contained only a few SOX9-positive nuclei or prevalent cytoplasmic staining, suggesting that most would have developed as ovaries given a longer culture period, though cortical enrichment of β -catenin was not yet apparent in most cases (Fig. 1C). The correlation in SOX9 staining intensity between gonad pairs was highly significant ($r=0.894$; $p<0.0001$). Synchronization between gonad pairs indicates that their sexual differentiation trajectory was either fixed by a common embryo/yolk-derived signal before stage 17 or determined by a shared genetic predisposition. However, this result argues against an environmental mechanism that relies on systemic communication after stage 17 to coordinate gonad fate.

These conclusions were supported by a second experiment performed in parallel with the PvT cultures. Here, eight embryos were incubated at the PvT until stage 17, at which point the gonad pair was dissected from each embryo, and the gonads were divided into two culture dishes, one of which was cultured at the MPT and the other at the FPT (Fig. 2A). Previous studies indicated that gonads isolated from stage 16.5 MPT or FPT embryos and cultured at the opposite temperature undergo sex reversal, similar to intact eggs shifted at stage 17 (Wibbels et al., 1991; Shoemaker-Daly et al., 2010). We therefore predicted that the fate of these gonads would be fully plastic: gonads shifted from PvT→MPT would develop as testes, whereas the gonads shifted to the FPT would develop as ovaries.

Contrary to expectations, in five of the seven surviving pairs, the gonad shifted to the MPT showed high SOX9 expression, as expected, but intermediate (2/5) to low (3/5) SOX9 levels were observed in the gonad shifted to the FPT (Fig. 2B and D). In the other two pairs, the gonad shifted to the FPT showed high cortical β -catenin and no SOX9 expression, and very weak SOX9 staining was observed in its partner at the MPT (Fig. 2C and D). While this was a small sample size, we propose that the first group was predisposed towards the male fate and the second group towards the female fate. This interpretation could explain why it was difficult to shift gonads from the first group to the female fate and gonads from the second group to the male fate. We predict that a longer culture duration would have resulted in full sex reversal, as previously observed in gonads shifted from FPT→MPT or MPT→FPT and cultured for up to 20 days (Moreno-Mendoza et al., 2001; Shoemaker-Daly et al., 2010).

Establishment of sexual fate early in the TSP

One interpretation of our finding that sexual fate is coordinated in the absence of systemic communication between the gonads is that sexual trajectory was already determined prior to the beginning of the culture period (stage 17) – by either an early-acting environmental signal or a genetic mechanism – and maintained along the same trajectory thereafter. We reasoned that if this were the case, then incubation at the MPT or FPT prior to a shift to the PvT at stage 17 should fix the sexual trajectory. To determine whether a sexual trajectory established prior to stage 17 is stable in the absence of an ongoing temperature signal, we incubated cohorts of 50 eggs at the MPT or FPT up to stage 17 and then shifted them *in ovo* to the PvT until just prior to hatching (stage 26). Because the PvT is equally permissive and unbiased to male and female development, we predicted that if sexual fate were stably determined by an environmental influence prior to stage 17, the initial incubation at the extreme temperature would decide the sex of the embryos. In this case, we expected that the MPT→PvT and FPT→PvT embryos would develop as males and females, respectively, as opposed to a null hypothesis of randomization of sexual fate within each group.

Table 1

Sexual fate is generally maintained at the PvT once established at the MPT or FPT during early gonad development.

Gonadal sex	MPT preincubation	FPT preincubation
Male	100.00% (22/22)	17.24% (5/29)
Female	0.00% (0/22)	72.41% (21/29)
Intersex	0.00% (0/22)	10.34% (3/29)
p-value	< 0.001	0.0241

In line with this reasoning, the vast majority of embryos developed according to their pre-incubation temperature after being shifted to the PvT at stage 17. Among the surviving embryos incubated at the FPT prior to stage 17, most (~72%; $n=21/29$) remained female ($p=0.0241$), ~17% ($n=5/29$) sex-reversed to male, and ~10% (3/29) were indistinguishable as either male or female. The eggs that were incubated at the MPT until stage 17 all developed as males ($n=22/22$), without a single sex reversal ($p<0.001$) (Table 1). These data suggest that after early temperature-based sexual biasing, randomization of sexual fate does not occur, even when the temperature influence is released at stage 17.

Discussion

How sex is determined at the pivotal temperature in a TSD species represents a potential intersection between environmental and genetic mechanisms of sex determination. Some authors, notably Claude Pieau's group in the 1980s and 1990s, argued that because the PvT is equally permissive to male and female development, genetic influences likely determine sex under these conditions (e.g., Zaborski et al., 1988; Pieau et al., 1998). Our finding that paired gonads cultured in isolation at the PvT adopt the same sexual fate supports the existence of cryptic genetic mechanisms operating at the PvT. Furthermore, experiments in which one gonad of a PvT embryo was shifted to the FPT and the other to the MPT also suggested a sexual predisposition shared by both gonads of the pair. These results also argue for an inherent bias acting in the embryo prior to stage 17. The co-existence of genetic and temperature-based systems has been documented in the dragon lizard *P. vitticeps* (Ezaz et al., 2005; Quinn et al., 2007), in the skink *B. duerreyi* (Shine et al., 2002; Radder et al., 2008; Quinn et al., 2009) and the turtle *A. mutica* (Valenzuela, 2008). Such overlapping systems could increase the fitness of the population across a wide or variable climate range.

While this sexual predisposition shared by the two gonads of a pair could be genetic, it could also potentially be attributed to a non-temperature-related environmental signal, such as a maternally-deposited factor in the yolk or stochastic variation in neuronal or other embryo-derived signals, to which the two nascent gonads were equally exposed prior to dissection (Janzen et al., 1998; Radder et al., 2009). In the painted turtle, inter-clutch variation in yolk steroid hormone levels has been correlated with clutch sex ratio at the PvT, i. e., clutches with higher β -estradiol levels at oviposition were more female-biased (Bowden et al., 2000). However, most reptiles exhibit minimal variation in steroid hormone levels within a given clutch (Conley et al., 1997; Janzen et al., 1998; Bowden et al., 2000), in line with the fact that all eggs of a clutch undergo vitellogenesis at the same time in the same maternal milieu (Callard et al., 1978; Bowden et al., 2002; Janzen et al., 2002). Consistent with this, efforts to correlate an individual's yolk hormone profile at oviposition with its sex at hatching after incubation at the PvT have not produced positive results (reviewed in Radder, 2007). However, influence from other non-steroidal factors in the yolk remains a possibility.

In our *in ovo* temperature shift experiment, pre-incubation under the influence of a strong polarizing thermal signal (at the MPT or FPT) prior to shifting to the PvT was sufficient to fix sexual trajectory in most cases. This experiment demonstrates that an early (prior to stage 17) sexual trajectory set by temperature is largely stable against randomization at the PvT, even while the temperature-sensitive window is still open and sexual fate is still plastic. In agreement with early temperature shift studies with *T. scripta* eggs, these experiments also suggest a discrepancy between the male and female programs. Previous publications suggest that in *T. scripta*, as well as the logger-head turtle *Caretta caretta*, the leopard gecko *Eublepharis macularius* and the alligator *A. mississippiensis*, the female fate becomes irreversibly set at the FPT at an earlier stage than the male fate at the MPT (Yntema and Mrosovsky, 1982; Bull, 1987; Deeming and Ferguson, 1988; Joss, 1989; Wibbels et al., 1991). The opposite phenomenon is observed in the turtles *E. orbicularis* and *Graptemys ouchitensis*, where the male fate becomes irreversible during MPT incubation earlier than the female fate induced by the FPT (Bull and Vogt, 1981; Pieau and Dorizzi, 1981). Other studies have shown that the sex-determining effects of temperature during the “primary sensitive period” (stages 16–22) of emydid turtles is dominant over effects derived from exposure before or after this period. In these studies, eggs were shifted between the MPT and FPT, and the incidence of sex reversal decreased as the eggs were shifted at progressively later stages (Bull and Vogt, 1981; Bull, 1987). In the current study, eggs were not shifted between temperatures that produce a single sex, but rather from one extreme to a temperature that is equally permissive to both. Furthermore, they were shifted at a stage that previous studies suggest is highly likely to result in sex reversal (Wibbels et al., 1991). The fact that both temperature-induced sexual fates, set at the MPT and FPT before stage 17, are mostly stable at the PvT is novel and supports the idea that whatever bias is established prior to stage 17 can be maintained independently of thermal influence. Pre-incubation at the MPT prior to stage 17 was sufficient to canalize the male pathway when eggs were shifted to the PvT; however, this was not true for about 1/4 of embryos pre-incubated at the FPT, which did not maintain female fate but instead developed as males or intersexes. Additional experiments, including a recalibration of the PvT with a larger sample size under our laboratory conditions, would be necessary to draw significant conclusions about the relative stability of the male vs. female programs.

Cryptic genetic mechanisms operating in TSD species

If genetic factors do determine sex at the PvT, they would likely have weaker effects than the dominant (e.g., Sry in mammals) or dosage-linked (*DMRT1* in birds) determinants present in GSD systems, as they can so easily be overridden by temperature. Interestingly, in the TSD European pond turtle *E. orbicularis*, Pieau et al. (1998) found that gonads of embryos incubated at the PvT showed the first signs of morphological differentiation later than stage-matched MPT/FPT embryos, suggesting that without the driving influence of temperature, the sex-specific transcriptional programs take longer to manifest. Indeed, if we consider sex as a threshold trait in which a male- or female-promoting signal must reach a particular level to sway the system towards one of two dichotomous outcomes (male vs. female) (Mittwoch, 2006; Quinn et al., 2011) and repress the alternative outcome (Kim et al., 2006), only a slight genetic predisposition in favor of the male or female program might be sufficient to determine sex in the absence of a strong temperature signal. This predisposition could potentially be polygenic and consist of genetic variants at multiple loci that collectively determine whether the male or female signal meets

the required threshold. There is evidence for such a mechanism operating in zebrafish (Liew et al., 2012).

Another possibility is that TSD species harbor cryptic, undifferentiated sex chromosomes carrying dominant or dosage-dependent regulators. While karyotyping analyses have not distinguished sex chromosomes in *T. scripta* (Stock, 1972; Bickham and Baker, 1976), many reptiles with GSD have homomorphic or micro sex chromosomes that cannot easily be distinguished by standard methods (Ezaz et al., 2005, 2006; Martinez et al., 2008; Badenhorst et al., 2013). If cryptic heterogamety is indeed present in TSD species, populations of TSD species could potentially contain many individuals in which the sexual genotype (e.g., ZZ or ZW) is discordant with the sexual phenotype (ovaries or testes). If such individuals suffered a significant fitness detriment (as they do in mammals, where similarly sex-reversed individuals are often sterile), we presume that the species would evolve away from temperature sensitivity and towards a GSD mechanism. As TSD is clearly a successful strategy, we should instead conclude that the potential genotype-phenotype discordance – if it exists – does not carry a fitness cost. This is consistent with the Charnov–Bull model for TSD (Charnov and Bull, 1977), which predicts that there must be a fitness benefit to producing a given sex at a particular temperature (i.e., females at high temperatures). To be maintained in the population, this benefit would likely have to supersede any potential detriment derived from genotype-phenotype incongruities.

Timing of sex determination

Our culture experiments indicate that sexual fate is coordinated between the two gonads of a PvT embryo by shared genetic or yolk-derived information rather than by a stochastic or systemic signal acting during the latter part of the bipotential period of gonad development. In addition, our temperature shift studies suggest that sexual trajectory is set and can be independently maintained well before the end of the TSP, many days prior to the onset of morphological differentiation into testes or ovaries. Male fate may be more strongly canalized by stage 17 at the MPT than is the female fate at the same stage at the FPT (Table 1). However, in both cases, the sexual trajectories established prior to stage 17 are usually perpetuated thereafter unless the gonads/eggs are shifted to an extreme temperature. Even in cases where the two gonads from an embryo incubated at PvT were incubated at two different temperatures after stage 17, they showed signs of a lingering predisposition towards a single fate, at least for the first week of the culture period (Fig. 2).

A handful of candidate sex-determining genes have been shown to be differentially expressed at the MPT and FPT as early as stage 16 (Ramsey and Crews, 2007; Ramsey et al., 2007; Rhen et al., 2007; Shoemaker et al., 2007a, 2007b; Smith et al., 2008), but the degree to which these differences reflect sex determination or establishment of a sexual differentiation trajectory has been difficult to evaluate, given the ease with which embryos can be sex-reversed until the end of the TSP (Wibbels et al., 1991). Our findings suggest that these early gene expression differences reflect the adoption of a self-sustaining sexual differentiation trajectory early in the TSP. Ongoing studies in our lab using RNA-seq analysis will examine the full extent of the differences between the male and female transcriptional programs as they operate at the MPT, FPT and PvT during the TSP. In addition, we are investigating genomic diversity in *T. scripta* males and females to identify potential regions involved in generating sexual bias in the absence of a strong thermal influence. This work, coupled with other emerging genomic resources for multiple TSD species (St John et al., 2012; Kaplinsky et al., 2013; Shaffer et al., 2013; Wang et al., 2013), will substantially help in understanding TSD at a mechanistic as well as an evolutionary level.

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References

- Aresco, M.J., 2004. Reproductive ecology of *Pseudemys floridana* and *Trachemys scripta* (Testudines: Emydidae) in northwestern Florida. *J. Herpetol.* 38, 249–256.
- Badenhorst, D., Stanyon, R., Engstrom, T., Valenzuela, N., 2013. A ZZ/ZW micro-chromosome system in the spiny softshell turtle, *Apalone spinifer*, reveals an intriguing sex chromosome conservation in Trionychidae. *Chromosome Res.* 21, 137–147.
- Barske, L.A., Capel, B., 2008. Blurring the edges in vertebrate sex determination. *Curr. Opin. Genet. Dev.* 18, 499–505.
- Barske, L.A., Capel, B., 2010. Estrogen represses SOX9 during sex determination in the red-eared slider turtle *Trachemys scripta*. *Dev. Biol.* 341, 305–314.
- Bickham, J.W., Baker, R.J., 1976. Chromosome homology and evolution of emydid turtles. *Chromosoma* 54, 201–219.
- Bowden, R.M., Ewert, M.A., Freedberg, S., Nelson, C.E., 2002. Maternally derived yolk hormones vary in follicles of the painted turtle, *Chrysemys picta*. *J. Exp. Zool.* 293, 67–72.
- Bowden, R.M., Ewert, M.A., Nelson, C.E., 2000. Environmental sex determination in a reptile varies seasonally and with yolk hormones. *Proc. Biol. Sci.* 267, 1745–1749.
- Bull, J.J., 1987. Temperature-sensitive periods of sex determination in a lizard—similarities with turtles and crocodilians. *J. Exp. Zool. A Ecol. Genet. Physiol.* 241, 143–148.
- Bull, J.J., Vogt, R.C., 1981. Temperature-sensitive periods of sex determination in emydid turtles. *J. Exp. Zool. A Ecol. Genet. Physiol.* 218, 435–440.
- Callard, I.P., Lance, V., Salhanick, A.R., Barad, D., 1978. The annual ovarian cycle of *Chrysemys picta*: correlated changes in plasma steroids and parameters of vitellogenesis. *Gen. Comp. Endocrinol.* 35, 245–257.
- Carpenter, A.E., Jones, T.R., Lamprecht, M.R., Clarke, C., Kang, I.H., Friman, O., Guertin, D.A., Chang, J.H., Lindquist, R.A., Moffat, J., Golland, P., Sabatini, D.M., 2006. CellProfiler: image analysis software for identifying and quantifying cell phenotypes. *Genome Biol.* 7, R100.
- Charnier, M., 1966. Action of temperature on the sex ratio in the *Agama agama* (Agamidae Lacertili) embryo. *CR Soc. Biol.* 160, 620–622.
- Charnov, E.L., Bull, J., 1977. When is sex environmentally determined? *Nature* 266, 828–830.
- Conley, A.J., Elf, P., Corbin, C.J., Dubowsky, S., Fivizzani, A., Lang, J.W., 1997. Yolk steroids decline during sexual differentiation in the alligator. *Gen. Comp. Endocrinol.* 107, 191–200.
- Deeming, D.C., Ferguson, M.W.J., 1988. Environmental regulation of sex determination in reptiles. *Philos. Trans. R. Soc. London* 322, 19–39.
- Engel, W., Klemme, B., Schmid, M., 1981. H-Y-antigen and sex-determination in turtles. *Differentiation* 20, 152–156.
- Ewert, M.A., Jackson, D.R., Nelson, C.E., 1994. Patterns of temperature-dependent sex determination in turtles. *J. Exp. Zool. A Ecol. Genet. Physiol.* 270, 3–15.
- Ezaz, T., Quinn, A.E., Miura, I., Sarre, S.D., Georges, A., Marshall Graves, J.A., 2005. The dragon lizard *Pogona vitticeps* has ZZ/ZW micro-sex chromosomes. *Chromosome Res.* 13, 763–776.
- Ezaz, T., Valenzuela, N., Grutzner, F., Miura, I., Georges, A., Burke, R.L., Graves, J.A., 2006. An XX/XY sex microchromosome system in a freshwater turtle, *Chelodina longicollis* (Testudines: Chelidae) with genetic sex determination. *Chromosome Res.* 14, 139–150.
- Graves, J.A.M., Shetty, S., 2001. Sex from W to Z: evolution of vertebrate sex chromosomes and sex determining genes. *J. Exp. Zool. A Ecol. Genet. Physiol.* 290, 449–462.
- Greenbaum, E., 2002. A standardized series of embryonic stages for the emydid turtle *Trachemys scripta*. *Can. J. Zool.* 80, 1350–1370.
- Janzen, F.J., Krenz, J.G., 2004. Phylogenetics: which was first, TSD or GSD? In: Valenzuela, N., Lance, V.A. (Eds.), *Temperature-Dependent Sex Determination in Vertebrates*, vi. Smithsonian Books, Washington D.C., pp. 121–130.
- Janzen, F.J., Phillips, P.C., 2006. Exploring the evolution of environmental sex determination, especially in reptiles. *J. Evol. Biol.* 19, 1775–1784.
- Janzen, F.J., Wilson, M.E., Tucker, J.K., Ford, S.P., 1998. Endogenous yolk steroid hormones in turtles with different sex-determining mechanisms. *Gen. Comp. Endocrinol.* 111, 306–317.
- Janzen, F.J., Wilson, M.E., Tucker, J.K., Ford, S.P., 2002. Experimental manipulation of steroid concentrations in circulation and in egg yolks of turtles. *J. Exp. Zool.* 293, 58–66.
- Joss, J.M.P., 1989. Gonadal development and differentiation in *Alligator mississippiensis* at male and female producing incubation temperatures. *J. Zool.* 218, 679–687.
- Kaplinsky, N.J., Gilbert, S.F., Cebra-Thomas, J., Lillevali, K., Saare, M., Chang, E.Y., Edelman, H.E., Frick, M.A., Guan, Y., Hammond, R.M., Hampilos, N.H., Opoku, D., S., Sariahmed, K., Sherman, E.A., Watson, R., 2013. The embryonic transcriptome of the red-eared slider turtle (*Trachemys scripta*). *PLoS One* 8, e66357.
- Kawai, A., Nishida-Umehara, C., Ishijima, J., Tsuda, Y., Ota, H., Matsuda, Y., 2007. Different origins of bird and reptile sex chromosomes inferred from comparative mapping of chicken Z-linked genes. *Cytogenet. Genome Res.* 117, 92–102.
- Kim, Y., Kobayashi, A., Sekido, R., DiNapoli, L., Brennan, J., Chaboissier, M.C., Poulat, F., Behringer, R.R., Lovell-Badge, R., Capel, B., 2006. *Fgf9* and *Wnt4* act as antagonistic signals to regulate mammalian sex determination. *PLoS Biol.* 4, e187.
- Liew, W.C., Bartfai, R., Lim, Z., Sreenivasan, R., Siegfried, K.R., Orban, L., 2012. Polygenic sex determination system in zebrafish. *PLoS One* 7, e34397.
- Marlen, M.D., Fischer, R.U., 1999. Parental investment in the red-eared slider turtle, *Trachemys scripta elegans*. *J. Herpetol.* 33, 306–309.
- Martineau, J., Nordqvist, K., Tilmann, C., Lovell-Badge, R., Capel, B., 1997. Male-specific cell migration into the developing gonad. *Curr. Biol.* 7, 958–968.
- Martinez, P.A., Ezaz, T., Valenzuela, N., Georges, A., Marshall Graves, J.A., 2008. An XX/XY heteromorphic sex chromosome system in the Australian chelid turtle *Emydura macquarii*: a new piece in the puzzle of sex chromosome evolution in turtles. *Chromosome Res.* 16, 815–825.
- Matsumoto, Y., Yatsu, R., Taylor, C., Crews, D., 2013. Changes in gonadal gene network by exogenous ligands in temperature-dependent sex determination. *J. Mol. Endocrinol.* 50, 389–400.
- Mittwoch, U., 2006. Sex is a threshold dichotomy mimicking a single gene effect. *Trends Genet.* 22, 96–100.
- Moreno-Mendoza, N., Harley, V.R., Merchant-Larios, H., 2001. Temperature regulates SOX9 expression in cultured gonads of *Lepidochelys olivacea*, a species with temperature sex determination. *Dev. Biol.* 229, 319–326.
- Mork, L., Capel, B., 2013. Conserved action of beta-catenin during female fate determination in the red-eared slider turtle. *Evol. Dev.* 15, 96–106.
- Mrosovsky, N., Pieau, C., 1991. Transitional range of temperature, pivotal temperatures and thermosensitive stages for sex determination in reptiles. *Amphibia-Reptilia* 12, 169–179.
- Nakamura, D., Wachtel, S.S., Lance, V., Becak, W., 1987. On the evolution of sex determination. *Proc. R. Soc. London, Ser. B* 232, 159–180.
- Pieau, C., Dorizzi, M., 1981. Determination of temperature sensitive stages for sexual differentiation of the gonads in embryos of the turtle, *Emys orbicularis*. *J. Morphol.* 170, 373–382.
- Pieau, C., Dorizzi, M., Richard-Mercier, N., 1999. Temperature-dependent sex determination and gonadal differentiation in reptiles. *Cell. Mol. Life Sci.* 55, 887–900.
- Pieau, C., Dorizzi, M., Richard-Mercier, N., Desvages, G., 1998. Sexual differentiation of gonads as a function of temperature in the turtle *Emys orbicularis*: endocrine function, intersexuality and growth. *J. Exp. Zool.* 281, 400–408.
- Pokorna, M., Kratochvil, L., 2009. Phylogeny of sex-determining mechanisms in squamate reptiles: are sex chromosomes an evolutionary trap? *Zool. J. Linn. Soc.* 156, 168–183.
- Quinn, A.E., Georges, A., Sarre, S.D., Guarino, F., Ezaz, T., Graves, J.A., 2007. Temperature sex reversal implies sex gene dosage in a reptile. *Science* 316, 411.
- Quinn, A.E., Radder, R.S., Sarre, S.D., Georges, A., Ezaz, T., Shine, R., 2009. Isolation and development of a molecular sex marker for *Bassiana duperreyi*, a lizard with XX/XY sex chromosomes and temperature-induced sex reversal. *Mol. Genet. Genom.* 281, 665–672.
- Quinn, A.E., Sarre, S.D., Ezaz, T., Marshall Graves, J.A., Georges, A., 2011. Evolutionary transitions between mechanisms of sex determination in vertebrates. *Biol. Lett.* 7, 443–448.
- Radder, R.S., 2007. Maternally derived egg yolk steroid hormones and sex determination: review of a paradox in reptiles. *J. Biosci.* 32, 1213–1220.
- Radder, R.S., Pike, D.A., Quinn, A.E., Shine, R., 2009. Offspring sex in a lizard depends on egg size. *Curr. Biol.* 19, 1102–1105.
- Radder, R.S., Quinn, A.E., Georges, A., Sarre, S.D., Shine, R., 2008. Genetic evidence for co-occurrence of chromosomal and thermal sex-determining systems in a lizard. *Biol. Lett.* 4, 176–178.
- Ramsey, M., Crews, D., 2007. Steroid signaling system responds differently to temperature and hormone manipulation in the red-eared slider turtle (*Trachemys scripta elegans*), a reptile with temperature-dependent sex determination. *Sex. Dev.* 1, 181–196.
- Ramsey, M., Shoemaker, C., Crews, D., 2007. Gonadal expression of Sf1 and aromatase during sex determination in the red-eared slider turtle (*Trachemys scripta*), a reptile with temperature-dependent sex determination. *Differentiation* 75, 978–991.
- Rhen, T., Metzger, K., Schroeder, A., Woodward, R., 2007. Expression of putative sex-determining genes during the thermosensitive period of gonad development in the snapping turtle, *Chelydra serpentina*. *Sex. Dev.* 1, 255–270.
- Sarre, S.D., Georges, A., Quinn, A., 2004. The ends of a continuum: genetic and temperature-dependent sex determination in reptiles. *Bioessays* 26, 639–645.
- Shaffer, H.B., Minx, P., Warren, D.E., Shedlock, A.M., Thomson, R.C., Valenzuela, N., Abramyan, J., Amemiya, C.T., Badenhorst, D., Biggar, K.K., Borchert, G.M., Botka, C. W., Bowden, R.M., Braun, E.L., Bronikowski, A.M., Bruneau, B.G., Buck, L.T., Capel, B., Castoe, T.A., Czerwinski, M., Delehaunty, K.D., Edwards, S.V., Fronick, C.C., Fujita, M. K., Fulton, L., Graves, T.A., Green, R.E., Haerty, W., Hariharan, R., Hernandez, O., Hillier, L.W., Holloway, A.K., Janes, D., Janzen, F.J., Kandoth, C., Kong, L., de Koning, A.J., Li, Y., Litterman, R., McLaughlin, M., Paiz, R.T., Pollock, D.D., Ponting, C.P., Radhakrishnan, S., Raney, B.J., Richman, J.M., St John, J., Schwartz, T., Sethuraman, A., Spinks, P.Q., Storey, K.B., Thane, N., Vinar, T., Zimmerman, L.M., Warren, W.C., Mardis, E.R., Wilson, R.K., 2013. The western painted turtle genome, a model for the evolution of extreme physiological adaptations in a slowly evolving lineage. *Genome Biol.* 14, R28.

- Shine, R., Elphick, M.J., Donnellan, S., 2002. Co-occurrence of multiple, supposedly incompatible modes of sex determination in a lizard population. *Ecol. Lett.* 5, 486–489.
- Shoemaker-Daly, C.M., Jackson, K., Yatsu, R., Matsumoto, Y., Crews, D., 2010. Genetic network underlying temperature-dependent sex determination is endogenously regulated by temperature in isolated cultured *Trachemys scripta* gonads. *Dev. Dyn.* 239, 1061–1075.
- Shoemaker, C., Ramsey, M., Queen, J., Crews, D., 2007a. Expression of *Sox9*, *Mis*, and *Dmrt1* in the gonad of a species with temperature-dependent sex determination. *Dev. Dyn.* 236, 1055–1063.
- Shoemaker, C.M., Queen, J., Crews, D., 2007b. Response of candidate sex-determining genes to changes in temperature reveals their involvement in the molecular network underlying temperature-dependent sex determination. *Mol. Endocrinol.* 21, 2750–2763.
- Smith, C.A., Shoemaker, C.M., Roeszler, K.N., Queen, J., Crews, D.P., Sinclair, A.H., 2008. Cloning and expression of *R-Spondin1* in different vertebrates suggests a conserved role in ovarian development. *BMC Dev. Biol.* 8, 72.
- St John, J.A., Braun, E.L., Isberg, S.R., Miles, L.G., Chong, A.Y., Gongora, J., Dalzell, P., Moran, C., Bed'hom, B., Abzhinov, A., Burgess, S.C., Cooksey, A.M., Castoe, T.A., Crawford, N.G., Densmore, L.D., Drew, J.C., Edwards, S.V., Faircloth, B.C., Fujita, M.K., Greenwold, M.J., Hoffmann, F.G., Howard, J.M., Iguchi, T., Janes, D.E., Khan, S.Y., Kohn, S., de Koning, A.J., Lance, S.L., McCarthy, F.M., McCormack, J.E., Merchant, M.E., Peterson, D.G., Pollock, D.D., Pourmand, N., Raney, B.J., Roessler, K.A., Sanford, J.R., Sawyer, R.H., Schmidt, C.J., Triplett, E.W., Tuberville, T.D., Venegas-Anaya, M., Howard, J.T., Jarvis, E.D., Guillelte Jr, L.J., Glenn, T.C., Green, R.E., Ray, D.A., 2012. Sequencing three crocodilian genomes to illuminate the evolution of archosaurs and amniotes. *Genome Biol.* 13, p. 415.
- Stock, A.D., 1972. Karyological relationships in turtles (Reptilia-Chelonia). *Can. J. Genet. Cytol.* 14, 859–868.
- Valenzuela, N., 2008. Relic thermosensitive gene expression in a turtle with genotypic sex determination. *Evol. Int. J. Org. Evol.* 62, 234–240.
- Wang, Z., Pascual-Anaya, J., Zadissa, A., Li, W., Niimura, Y., Huang, Z., Li, C., White, S., Xiong, Z., Fang, D., Wang, B., Ming, Y., Chen, Y., Zheng, Y., Kuraku, S., Pignatelli, M., Herrero, J., Beal, K., Nozawa, M., Li, Q., Wang, J., Zhang, H., Yu, L., Shigenobu, S., Liu, J., Flicek, P., Searle, S., Kuratani, S., Yin, Y., Aken, B., Zhang, G., Irie, N., 2013. The draft genomes of soft-shell turtle and green sea turtle yield insights into the development and evolution of the turtle-specific body plan. *Nat. Genet.* 45, 701–706.
- Wibbels, T., Bull, J.J., Crews, D., 1991. Chronology and morphology of temperature-dependent sex determination. *J. Exp. Zool.* 260, 371–381.
- Wibbels, T., Cowan, J., LeBoeuf, R., 1998. Temperature-dependent sex determination in the red-eared slider turtle, *Trachemys scripta*. *J. Exp. Zool.* 281, 409–416.
- Wibbels, T., Crews, D., 1995. Steroid-induced sex determination at incubation temperatures producing mixed sex ratios in a turtle with TSD. *Gen. Comp. Endocrinol.* 100, 53–60.
- Wolf, U., 1998. The serologically detected H-Y antigen revisited. *Cytogenet. Cell Genet.* 80, 232–235.
- Yntema, C.L., Mrosovsky, N., 1982. Critical periods and pivotal temperatures for sexual differentiation in loggerhead sea turtles. *Can. J. Zool.* 60, 1012–1016.
- Zaborski, P., Dorizzi, M., Pieau, C., 1982. H-Y antigen expression in temperature sex-reversed turtles (*Emys orbicularis*). *Differentiation* 22, 73–78.
- Zaborski, P., Dorizzi, M., Pieau, C., 1988. Temperature-dependent gonadal differentiation in the turtle *Emys orbicularis*: concordance between sexual phenotype and serological H-Y antigen expression at threshold temperature. *Differentiation* 38, 17–20.